

Dimerization of α -Chymotrypsin. II. Ionic Strength and Temperature Dependence*

Kirk C. Aune,[†] Lowell C. Goldsmith,[‡] and Serge N. Timasheff[§]

ABSTRACT: The acid dimerization equilibrium constant of α -chymotrypsin was determined by the method of sedimentation equilibrium as a function of ionic strength and temperature. It was found that this reaction is accompanied by the apparent preferential binding of salt, a negative change in heat capacity, and a positive entropy at low temperature. Change of medium from H₂O to D₂O enhanced the dimeriza-

tion. Perchlorate ions were found to strengthen dimer formation, while sulfate weakened it. Analysis of these thermodynamic observations led to the conclusions that water leaves the domain of the intermolecular contact region when the dimer forms and that the released water comes not only from hydrophobic sites, but also from the hydration shells of charged groups in the contact region.

In the preceding study (Aune and Timasheff, 1971) an analysis of the pH dependence of the dimerization of α -chymotrypsin below pH 5.5 revealed that it could be completely accounted for in terms of two specific interacting charged groups in the dimer. These groups were identified as being most likely the imidazole group of histidine 57 and the α -carboxyl group of tyrosine 146.

In earlier studies, Steiner (1954) and Egan *et al.* (1957) had found that an increase in ionic strength enhanced the association of α -chymotrypsin in the acid pH region. Steiner (1954) interpreted this effect in terms of the diminution of unfavorable electrostatic repulsion. The temperature dependence of turbidity at a single protein concentration further led Steiner (1954) to conclude that the enthalpy of dimerization was very small or zero. In the present study, it has been found that the nonspecific ionic strength effect might be

of minor importance, the predominant effect of salt being a change in preferential solvation in the contact region of the dimer. This change is reflected in a temperature dependence of the association. The significance of these observations to the mechanism of α -chymotrypsin dimerization is discussed in this paper.

Materials and Methods

The deuterium oxide used in this study was Bio-Rad¹ Lot No. 7056. The density and viscosity of deuterium oxide used were those compiled by Kirshenbaum (1951). The sedimentation coefficient of α -chymotrypsin was measured in the conventional manner on a Spinco Model E analytical ultracentrifuge at 56,000 rpm. The schlieren patterns as a function of time were recorded on Kodak metallographic plates and peak positions were measured on a Nikon 6C microcomparator. The least-squares slope of the natural logarithm of the peak radial position *vs.* time was computed and the results were corrected according to Svedberg and Pederson (1940) to the conditions of hydrogen oxide at 20° on a Wang 370 electronic programmable calculator. The partial specific volume of α -chymotrypsin in deuterium oxide was assumed to be lowered by a factor of $1/1.0155$ relative to that in hydrogen oxide, because of hydrogen exchange without volume change (Hvidt and Nielson, 1966; Edelstein and

* From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154, Publication No. 766, and the Pioneering Research Laboratory, Eastern Marketing and Nutrition Research Division, Agricultural Research Service, U. S. Department of Agriculture at Brandeis University, Waltham, Massachusetts 02154. Received September 16, 1970. This work was supported in part by the National Institutes of Health, Grant No. GM 14603, and the National Science Foundation, Grant No. GB 12619.

[†] Recipient of American Cancer Society Postdoctoral Fellowship No. PF-484; present address: Department of Biochemistry and Molecular Biology, The Ohio State University, Columbus, Ohio 43210.

[‡] Carl Herzog Fellow of the American Dermatological Association.

[§] To whom correspondence should be addressed at the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. 02154

¹ Mention of this and other companies or products is for the convenience of the reader and does not constitute an endorsement by the U. S. Department of Agriculture.

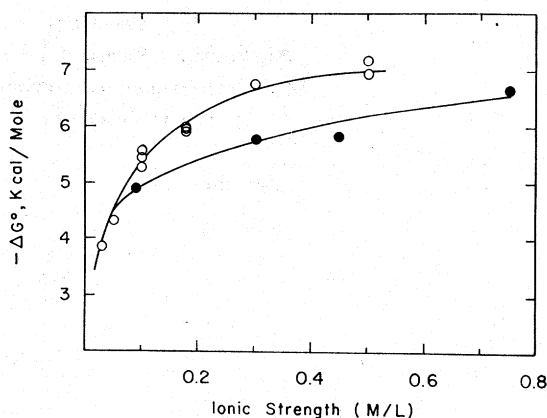


FIGURE 1: Dependence of the free energy of α -chymotrypsin dimerization on ionic strength. Open circles are data in NaCl; filled circles are data in CaCl_2 . Conditions: 25°, 0.01 M acetate buffer, pH 4.1 and 4.0, for NaCl and CaCl_2 , respectively.

Schachman, 1967). This resulted in a correction of $s_{20,w}$ of ca. 6.5%. The mass increase in deuterium oxide should increase the absolute value of $s_{20,w}$ by only ca. 1%. The temperature coefficient of the partial specific volume of α -chymotrypsin, \bar{v} , was taken to be the same as that of bovine serum albumin (Hunter, 1967), so that: $\bar{v} = 0.736 - 0.00035(t - 25)$, where t is the temperature in degrees Celsius. The operation of the ultracentrifuge at temperatures above 30° was facilitated by lining the wall of the chamber with a 0.020-in. thick flashing aluminum cylinder. With the aluminum cylinder in place, the elevated temperatures could be maintained even with the refrigeration turned on, so that no oil condensed on the lenses during the runs. All other experimental procedures have been described in the preceding paper (Aune and Timasheff, 1971).

The water and ion-pair activities of the various salt solutions were extracted from the data compiled by Robinson and Stokes (1959).

The association of α -chymotrypsin was also followed by sedimentation equilibrium experiments and the thermodynamic dimerization equilibrium constant was computed from the concentration distribution in the ultracentrifuge cell in the manner already described (Aune and Timasheff, 1971).

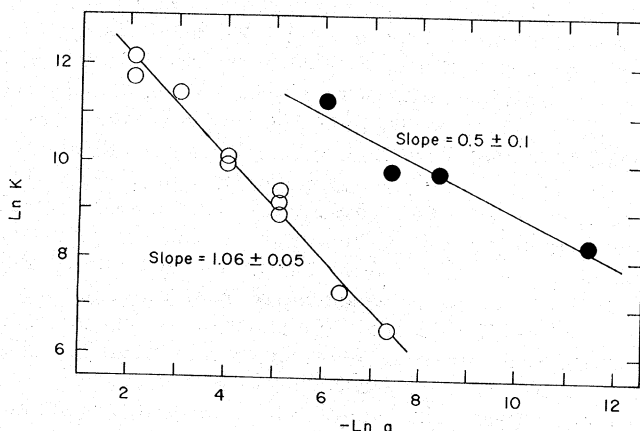


FIGURE 2: Dependence of the natural logarithm of the dimerization equilibrium constant on the natural logarithm of ion-pair activity. Open circles are NaCl; filled circles are CaCl_2 . Conditions: 25°, 0.01 M acetate buffer, pH 4.1 and 4.0 for NaCl and CaCl_2 , respectively.

Results and Discussion

The dependence on salt concentration of the acid dimerization of α -chymotrypsin was examined between 0.02 M and 0.50 M, for several ionic species. The resulting dependence of the free energy of dimerization at pH 4.1 on ionic strength is shown in Figure 1 for NaCl (upper curve) and CaCl_2 (lower curve). It is seen that for both salts, an increase in ionic strength enhances the dimerization reaction. The two curves are, however, displaced from each other, indicating that the observed effect cannot be interpreted solely in terms of nonspecific Debye-Hückel screening of electrostatic interactions, and that specific ion effects must be operative. Plotting the data as the logarithm of the equilibrium constant *vs.* the logarithm of the ion-pair activity gives a close to linear relationship, as shown in Figure 2. Recalling (Aune and Timasheff, 1971) that the equilibrium constant of dimerization can be expressed in terms of solution variables, the total differential, $d \ln K$, at constant pH is

$$d \ln K = \left(\frac{\partial \ln K}{\partial \ln a_X} \right)_{a_{\text{H}_2\text{O}}} d \ln a_X + \left(\frac{\partial \ln K}{\partial \ln a_{\text{H}_2\text{O}}} \right)_{a_X} d \ln a_{\text{H}_2\text{O}} \quad (1)$$

where the subscript X refers to supporting electrolyte. Using the Wyman equation (1964)

$$\left(\frac{\partial \ln K}{\partial \ln a_i} \right)_{a_{j \neq i}} = \Delta \bar{v}_i \quad (2)$$

and rearranging yields the expression

$$\frac{d \ln K}{d \ln a_X} = \Delta \bar{v}_X + \Delta \bar{v}_{\text{H}_2\text{O}} \frac{d \ln a_{\text{H}_2\text{O}}}{d \ln a_X} \quad (3)$$

Tanford (1969) has discussed this equation and has shown that, for multicomponent systems, the quantity, $d \ln K / d \ln a_X$, is the difference between preferential binding of solvent components, $\Delta \bar{v}_{\text{pref}}$, to the two end states of the reaction in question. The parameter, $\Delta \bar{v}_{\text{pref}}$, can be written as

$$\Delta \bar{v}_{\text{pref}} = \Delta \bar{v}_X - \frac{m_X}{m_{\text{H}_2\text{O}}} \Delta \bar{v}_{\text{H}_2\text{O}} \quad (4)$$

where m_i represents the molality of species *i*. Thus, the slopes of the lines in Figure 2 are the quantity, $\Delta \bar{v}_{\text{pref}}$. For a dimerization reaction, this quantity can be expressed as

$$\Delta \bar{v}_{\text{pref}} = \bar{v}_X^D - 2\bar{v}_X^M - \frac{m_X}{m_{\text{H}_2\text{O}}} (\bar{v}_{\text{H}_2\text{O}}^D - 2\bar{v}_{\text{H}_2\text{O}}^M) \quad (5)$$

where the superscripts D and M refer to the dimer and monomer, respectively.

The quantity, $\Delta \bar{v}_{\text{pref}}$, is found to be 1.06 for the sodium chloride data of Figure 2 and 0.5 for the calcium chloride data. Assuming that there is no change in hydration during dimerization, *i.e.*, that the quantity, $\bar{v}_{\text{H}_2\text{O}}^D - 2\bar{v}_{\text{H}_2\text{O}}^M$, is equal to zero, leads to the result that, at pH 4.1, dimer formation is accompanied by the binding of one sodium chloride ion pair or one-half of a calcium chloride. In solutions of electrolytes, the experimentally obtainable quantity is the ion-pair activity, namely, the combined activities of the

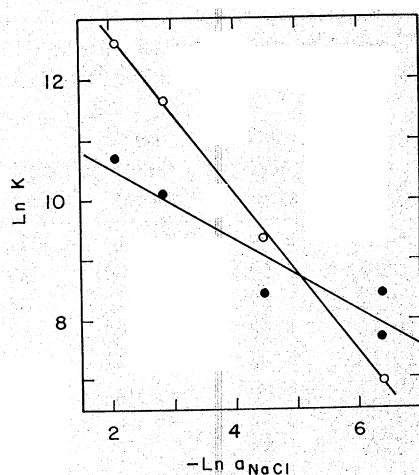


FIGURE 3: Dependence of the natural logarithm of the dimerization equilibrium constant on the natural logarithm of the sodium chloride ion-pair activity; 25°, 0.01 M acetate buffer; open circles: pH 3.40; filled circles: pH 5.10.

ions making up the neutral salt (Robinson and Stokes, 1959). Because of this, $\Delta\bar{\nu}_x$ is a measure of the interactions of both ions with the protein, even though the energies and mechanisms of interaction with a protein of the cation and anion of an ion pair may be quite different. For example, it is quite possible for one ion of a neutral salt to become strongly bound at a specific site on the protein, while the other ion interacts nonspecifically with the protein as a component of the Debye-Hückel double layer. Single ion activities can be derived only by making assumptions. Thus, if the binding of only one ion of a pair changes during the reaction, this change cannot be measured directly, but can be calculated in terms of a model. The quantity, $\Delta\bar{\nu}_x$, can be expressed in general form in terms of changes in binding of single ions, $\Delta\bar{\nu}_+$ and $\Delta\bar{\nu}_-$

$$\Delta\bar{\nu}_x = \frac{\Delta\bar{\nu}_+ \left(1 + \frac{n_-}{n} X\right) + \Delta\bar{\nu}_- \left(1 - \frac{n_+}{n} X\right)}{n} \quad (6)$$

where

$$X = \frac{d \ln (\gamma_+/\gamma_-)}{d \ln m} \quad (7)$$

and

$$n = n_+ + n_- \quad (8)$$

with n_+ and n_- representing the number of cations and anions per mole of neutral salt and γ the activity coefficient of the subscripted ionic species. As discussed elsewhere for the uniunivalent case (Aune and Tanford, 1969), the quantity, X , is usually considered to be zero. Inspection of eq 6 shows that nonintegral values of $\Delta\bar{\nu}_x$ are not impossible, since in the case of calcium chloride, a value of less than one could be obtained for $\Delta\bar{\nu}_x$, if less than three ions became bound during dimer formation.

In the preceding paper (Aune and Timasheff, 1971), it has been shown that the asymmetric dimer present in the α -chymotrypsin crystal (Matthews *et al.*, 1967) is most probably maintained in solution. This imposes the requirement

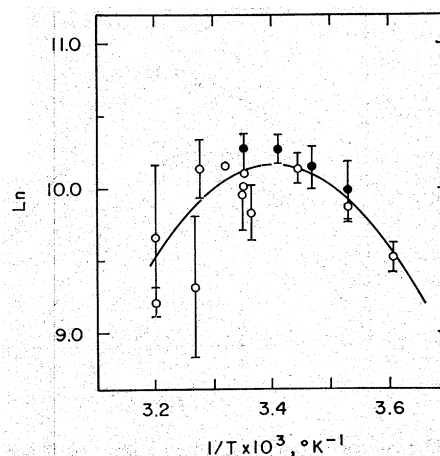


FIGURE 4: Dependence of the natural logarithm of the dimerization equilibrium constant on the reciprocal of the absolute temperature. Conditions: 0.178 M NaCl, 0.01 M acetate buffer, pH 4.10; filled circles are data obtained in 0.10 M NaCl, corrected to 0.178 M.

that the dyad axis of symmetry present in the crystal must also be satisfied by bound ions. A single bound ion or ion pair could not satisfy the dyad axis of symmetry unless it were located *directly on* the axis of symmetry. Since this is highly improbable, the binding of a minimum of two cations or two anions is required to explain the ionic strength dependence. Alternately more ions or ion pairs could become bound during dimerization, if $\Delta\bar{\nu}_{H_2O}$ had a positive value. As will be shown below, this is very unlikely.

An examination of $\Delta\bar{\nu}_{pref}$ as a function of pH for the case of NaCl has revealed that this quantity is pH dependent. As shown in Figure 3, the slopes of $\ln K$ vs. $\ln a_{NaCl}$ are 0.6 and 1.3 at pH 5.1 and 3.4, respectively. It is very improbable that the difference between the number of ions specifically bound to the interaction site in the monomer and dimer could be pH dependent. Since this would necessarily affect the pH dependence of the number of protons released during the reaction, it would require that the mechanism of dimerization also vary with pH. Such a requirement would obviate the simple interpretation of the observed pH dependence of α -chymotrypsin dimerization (Aune and Timasheff, 1971) in terms of the formation of two symmetrically located identical interprotein ion pairs. It seems most reasonable, then, that no change in specific ion binding is involved during dimerization, but rather that the observed $\Delta\bar{\nu}_{pref}$ reflects a nonzero value of $\Delta\bar{\nu}_{H_2O}$, namely a change in the degree of hydration of the protein during the association. The experimental results at pH 4.1 would then correspond to $\Delta\bar{\nu}_{H_2O}$ of about -240 moles of water per mole of dimer formed in sodium chloride and -270 in calcium chloride. Thus, approximately 125 water molecules must *leave* the domain of the contact region of *each* monomer when the dimer is formed. This is the maximal negative value for $\Delta\bar{\nu}_{H_2O}$; this quantity would become more positive by 125 water molecules for every mole of neutral salt bound during the association.

In order to resolve the question of the solvent species which are most important in the dimerization, the temperature dependence of this reaction was determined at pH 4.1. The results are shown in Figure 4, where the natural logarithm of the dimerization equilibrium constant is plotted vs. the reciprocal of the absolute temperature. The open circles are data obtained in 0.178 M NaCl while the data indicated

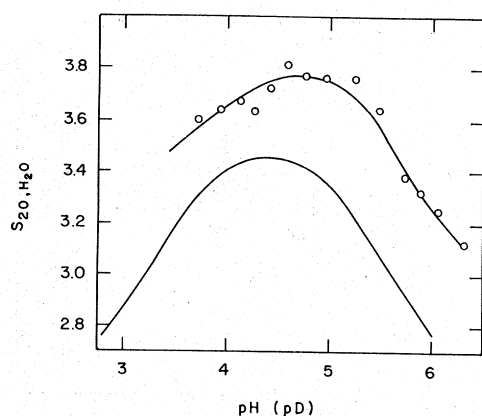


FIGURE 5: Dependence of the sedimentation coefficient, $s_{20,w}$, of α -chymotrypsin on pH (pD): open circles, D_2O , 0.15 M NaCl, 0.01 M acetate buffer, 25°. The lower curve is a theoretical curve calculated for α -chymotrypsin in H_2O under the same conditions (based on available sedimentation velocity data and equilibrium constants obtained from sedimentation equilibrium experiments). The protein concentration was 3.6 g/l.

by the filled circles were obtained in 0.1 M NaCl and corrected to 0.178 M. It is evident that the data do not follow a linear van't Hoff plot, meaning that either the enthalpy is temperature dependent or that separate processes are being measured at the low and high temperatures. Processes other than the dimerization could be the formation of covalent aggregates or autolysis of the enzyme. These seem to be ruled out by the observation that the protein concentration distribution in the ultracentrifuge cell can be reversed by lowering the temperature after an experiment at 40°.

Furthermore, autolysis is negligible after exposure for 60 hr to 30° at pH 5 (Kumar and Hein, 1970). It would, therefore, seem likely that minimal autolysis would occur at 40°, pH 4.1, since only a 50-fold increase in the second-order rate constant for autolysis is observed at pH 7.9 (Kumar and Hein, 1970) for a temperature increase from 30 to 40°. Therefore, the enthalpy of dimerization must be temperature dependent. Curve fitting of the data of Figure 4 yields a value for the change in heat capacity during dimerization, ΔC_p , of -700 ± 300 cal/(mole deg). The other derived thermodynamic parameters, namely the free energy, ΔG° , enthalpy, ΔH° , and entropy, ΔS° , are listed in Table I.

The dimerization is accompanied by a positive change in enthalpy and unitary entropy, ΔS_u° , at low temperatures as well as a negative heat capacity change. This is fully consistent with a hydrophobic interaction being important in the formation of the dimer. Namely, ordered water molecules around hydrophobic side chains in the monomer are driven out when dimer is formed. This loss of ordered water gives rise to the positive entropy and negative heat capacity difference.² The combination of the temperature-dependence and ionic strength dependence data support the conclusion that water is indeed leaving the contact region during dimer formation and that *no* specific ion binding accompanies this reaction.

² The small negative value of ΔS_u° at the highest temperature studied (35°) cannot be regarded as sufficient evidence for the ordering of water at higher temperatures. It must be recalled that the experimental values of ΔS_u° contain contributions from molecular events other than a change in the degree of water immobilization, for example, the decrease in the freedom of motion of the protein monomeric units when they associate.

TABLE I: Thermodynamic Parameters for the Dimerization of α -Chymotrypsin in 0.178 M NaCl–0.01 M HAc, pH 4.12.

T (°C)	$\ln K$	$-\Delta G^\circ$ (kcal/ mole)	ΔH° (kcal/ mole)	ΔS° (eu)	ΔS_u° (eu)
0.0	9.20	4.99	14.51	71	79
5.0	9.63	5.32	10.99	59	67
10.0	9.92	5.68	7.47	46	54
15.0	10.10	5.78	3.95	34	48
20.0	10.16	5.92	0.42	22	30
25.0	10.13	6.00	-3.09	10	18
30.0	9.99	6.02	-6.61	-2	6
35.0	9.77	5.98	-10.13	-13	-5

$$\Delta C_p \cong -700 \text{ cal/(mole deg)}$$

An independent approach to the problem of the role of water in the dimerization mechanism is to change the nature of the water itself, *i.e.*, to measure dimer formation in deuterium oxide. Interactions involving water (whether water–water or water–protein) should be different for H_2O and D_2O . Indeed solubility studies of nonpolar amino acids in D_2O have led to the conclusion that hydrophobic interactions should be stronger in that solvent than in H_2O (Kreshek *et al.*, 1965). Thus, if a hydrophobic interaction is involved in the dimerization of α -chymotrypsin, as suggested from the temperature-dependence study, D_2O should enhance that dimerization.

Exactly such an enhancement is observed, as shown in Figure 5, where $s_{20,w}$ is plotted *vs.* pH (pD). The circles represent experimental points obtained in D_2O at 25°, in 0.15 M NaCl, 0.01 M acetate buffer. The solid line represents calculated values of $s_{20,w}$ in H_2O under identical conditions from the pH dependence of the equilibrium constant, using values of 4.1 S and 2.5 S for $s_{20,w}^0$ of the dimer and monomer, respectively, and a value of 0.006 l/g for the concentration dependence constant, k , of the equation

$$s_{20,w} = s_{20,w}^0(1 - kc) \quad (9)$$

for both species. This calculated curve is consistent with scattered experimental values of $s_{20,w}$ obtained at various conditions, and corrected to these conditions with the established thermodynamic parameters of the association. It is clear that in D_2O , $s_{20,w}$ reaches higher values than in H_2O . The enhancement of the association is accompanied by a shift in the position of the pH (pD) maximum to higher values by about 0.6 unit. This is consistent with the expected shift of the pK 's of ionizing groups on a protein when transferred from H_2O to D_2O if the pK 's of these groups shift in the same manner as that of acetic acid (Glasoe and Long, 1960).

With the knowledge that both ionic and hydrophobic interactions are involved in α -chymotrypsin dimerization, further insight into the mechanism of this reaction was sought from experiments in solutions of salts which are near the extremes of the Hofmeister series, namely, sodium perchlorate and sodium sulfate. It is known that, in general, sodium perchlorate salts in proteins, while sodium sulfate salts them out (Von Hippel and Schleich, 1969). Extrapolating this to the dimerization reaction, it would have been expected

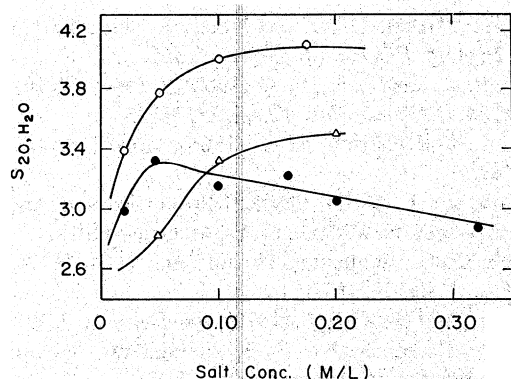


FIGURE 6: Dependence of the sedimentation coefficient, $s_{20,w}$, of α -chymotrypsin on salt concentration. Conditions: 25°, 0.01 M acetate buffer pH 4.3; open circles: NaClO_4 ; filled circles: Na_2SO_4 ; triangles: NaCl ; the protein concentration was 3.7 g/l.

that sodium perchlorate should favor monomer formation, while sodium sulfate should favor dimer formation. The results, shown in Figure 6, indicate that just the reverse is true. The sedimentation coefficient of α -chymotrypsin rises most rapidly with an increase in NaClO_4 concentration and least rapidly in the Na_2SO_4 system. The observations of Figure 6 were verified by sedimentation equilibrium experiments and their results are plotted as $\ln K$ vs. $\ln a_x$ in Figure 7. The data at low salt activities are somewhat uncertain since convective effects might be important at low salt concentrations. The results in sodium sulfate are difficult to interpret. In terms of ionic strength, Na_2SO_4 seems to be equivalent to NaCl in the low salt concentration range. At higher concentrations, however, sodium sulfate no longer appears to enhance the dimerization reaction as there is no further increase in $s_{20,w}$. This could reflect either a specific effect on the dimerization or a strong preferential solvation of the protein. Accounting for the difference in $s_{20,w}$ for α -chymotrypsin obtained in solutions of sodium chloride and sodium sulfate solely in terms of difference in preferential solvation (Aune and Timasheff, 1970) leads to the result that, in sodium sulfate, an additional 1 g of water must be bound to 1 g of protein in order to explain the difference in sedimentation properties at 0.2 M salt concentration. Since such a value of bound water is highly unlikely, the difference in $s_{20,w}$ must reflect, at least in part, a specific effect of the salt on the dimerization equilibrium, although some contribution from preferential solvation of the protein is also quite likely. The thermodynamic equilibrium constants determined in sodium sulfate solutions, shown in Figure 7, confirm the conclusion that dimerization was suppressed, although these values were not corrected for preferential solvation and, thus, contain a contribution from that effect.

The effect of sodium perchlorate is dramatic. The sedimentation coefficient rises rapidly to a value of close to 4.0 S at a salt concentration of 0.1 M and then appears to level off. At higher concentrations the protein is precipitated out of solution. The effects of sodium perchlorate and sodium sulfate are reversed from what would be expected from their salting in and out properties (Von Hippel and Schleich, 1969). The rules for salting in and out have been established, however, for the activity coefficient of the entire protein molecule (Scatchard and Kirkwood, 1932). In other words, the interaction of solvent with the entire surface of the protein molecule is important. In the case of a dimerization reaction, the interaction of solvent with the protein should be significantly

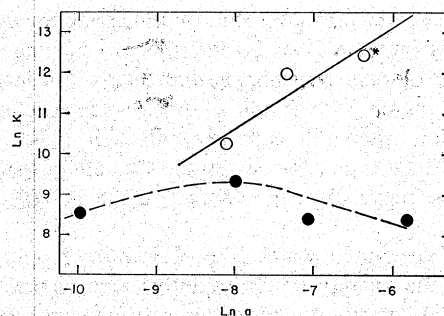


FIGURE 7: Dependence of the natural logarithm of the dimerization equilibrium constant on the natural logarithm of the ion-pair activity. Conditions: 25°, 0.01 M acetate buffer, pH 4.1; open circles: NaClO_4 ; filled circles: Na_2SO_4 ; asterisk indicates a data point obtained at pH 3.41 and corrected to pH 4.10.

affected only in the region of intermolecular contact. Thus, if this region were to differ appreciably from the general surface of the molecule, it is a necessary thermodynamic consequence that solvent interactions would result in favoring either the monomer or the dimer state.

Perchlorate ions are believed to salt out hydrophobic groups less effectively than sulfate ions (McDevit and Long, 1952; Schrier and Schrier, 1967; Von Hippel and Schleich, 1969). Thus, if hydrophobic interactions are present in the contact region, it would be expected that the association would be stronger in sodium sulfate than in sodium perchlorate. That such interactions are important in the dimerization of α -chymotrypsin has been demonstrated by the temperature dependence of the reaction and the experiments in D_2O . The results in the presence of perchlorate and sulfate ions then must mean that these solvents cannot act *only* at the hydrophobic regions. It must be concluded, therefore, that the solvent ions affect the interaction with water molecules of the charged groups on the protein in the contact region. The net effect must be that, in sodium sulfate, the contact region of the monomer molecules is more hydrated than in sodium perchlorate. The plausibility of this concept was verified by calculations of the mean activity coefficients of various salts in mixed electrolytes, according to the methods of Robinson and Stokes (1959). The results show that sodium fluoride (which is at the same end of the Hofmeister series as sodium sulfate) decreases the mean activity coefficient of a given salt more than sodium perchlorate at the same molality. This is consistent with the notion that the hydration shell around charged groups on the protein is more disrupted by sodium perchlorate than by sodium sulfate, with the result that the charge-charge interactions of the dimer (Aune and Timasheff, 1971) are stabilized.

Conclusions

The reaction for the dimerization of α -chymotrypsin as written, $2A \rightleftharpoons A_2$, is an extreme oversimplification of what is actually happening when the two protein molecules interact in a solution of water and ions. The complexity of the situation becomes quite evident when the reaction is analyzed under a variety of solvent conditions. The correlation of several thermodynamic measurements leads to the following conclusions concerning the mechanism of the dimerization of α -chymotrypsin.

1. At least one specific anionic-cationic interaction is formed on dimerization (Aune and Timasheff, 1971).

2. Part of the enhancement of dimerization when ionic strength is increased can be interpreted as being due to a diminution of long-range nonspecific electrostatic interactions.

3. The major part of the observed enhancement of the dimerization when salt concentration is increased must be attributed to the fact that a certain amount of "structured" water must move from the contact region in order to form the dimer.

4. The structured water is probably of two types: that found in the vicinity of hydrophobic side chains and that found in the vicinity of charged groups in the contact region.

Acknowledgment

The authors would like to thank Mrs. Valda Bolis and Miss Barbara Melander for their able assistance in carrying out the sedimentation velocity experiments in D₂O and several sedimentation equilibrium experiments.

References

- Aune, K. C., and Tanford, C. (1969), *Biochemistry* 8, 4586.
 Aune, K. C., and Timasheff, S. N. (1970), *Biochemistry* 9, 1481.
 Aune, K. C., and Timasheff, S. N. (1971), *Biochemistry* 10, 1609.
 Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem.* 242, 306.
 Egan, R., Michel, H. O., Schlueter, R., and Jandorf, B. J. (1957), *Arch. Biochem. Biophys.* 66, 366.
 Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem.* 64, 189.
 Hunter, M. (1967), *J. Phys. Chem.* 71, 3717.
 Hvidt, A., and Nielson, S. O. (1966), *Advan. Protein Chem.* 21, 287.
 Kirshenbaum, I. (1951), *Physical Properties and Analysis of Heavy Water*, New York, N. Y., McGraw-Hill.
 Kreshek, G. C., Schneider, H., and Scheraga, H. A. (1965), *J. Phys. Chem.* 69, 3132.
 Kumar, S., and Hein, G. E. (1970), *Biochemistry* 9, 291.
 Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature (London)* 214, 652.
 McDevit, W. F., and Long, F. A. (1952), *J. Amer. Chem. Soc.* 74, 1773.
 Robinson, R. A., and Stokes, R. H. (1959), *Electrolyte Solutions*, London, Butterworths.
 Scatchard, G., and Kirkwood, J. G. (1932), *Physik Z.* 33, 297.
 Schrier, E. E., and Schrier, E. B. (1967), *J. Phys. Chem.* 71, 1851.
 Steiner, R. F. (1954), *Arch. Biochem. Biophys.* 53, 457.
 Svedberg, T., and Pederson, K. O. (1940), *The Ultracentrifuge*, London, Oxford University Press.
 Tanford C. (1969), *J. Mol. Biol.* 39, 539.
 Von Hippel, P. H., and Schleich, T. (1969), in *Structure and Stability of Biological Macromolecules*, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Dekker, p 417.
 Wyman, J. (1964), *Advan. Protein Chem.* 19, 224.